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METHOD AND DISPOSABLE DEVICES FOR MICRO EXTRACTION

a TITLE OF THE INVENTION

The present invention relates generally to extraction technology.

a TECHNICAL FIELD

More specifically the invention relates, in a first aspect, to an apparatus having disposable elements for carrying out liquid-liquid micro extraction and liquid-liquid-liquid micro extraction.

The invention further relates to methods for liquid-liquid micro extraction and liquid-liquid-liquid micro extraction whereby there is obtained a high enrichment of analyte in the acceptor solution.

Finally, the invention relates to a special disposable device for use in liquid-liquid micro extraction.

With regard to liquid-liquid micro extraction, this relates especially to extraction of an analyte from an aqueous sample solution to an organic solvent where the analyte is enriched in the organic solvent.

Where the aforementioned liquid-liquid-liquid micro extraction is concerned, an analyte is extracted from an aqueous sample solution through a water immiscible liquid to an aqueous acceptor solution.

a BACKGROUND ART

Introduction

In capillary separation methods such as gas chromatography (GC), capillary electrophoresis (CE), capillary electrochromatography (CEC) and micro high performance liquid chromatography (HPLC) injection volumes are in the nl to µl range. Sample pre-treatment is necessary when these methods are used to determine analytes in complex matrices such as biological fluids. The principal objectives of sample pre-treatment involves concentration of the analytes to a concentration suitable for detection and removal of as many interfering compounds as possible. The use of an extraction technique is common in the pre-treatment of most types of samples. Sample extraction is the most tedious and time consuming step in the analysis of drugs present in the pg/ml to µg/ml range of biological fluids such as blood, serum, plasma, or urine.

No sample preparation technique is able to trap analytes in 1 - 50 μl of solvent for direct injection into the analytical instrument. Ideally the analytes should be trapped in an organic solvent for injection into a GC instrument and in an aqueous solvent for injection into a CE instrument or a micro HPLC instrument. The most frequently used extraction techniques are liquid-liquid extraction (LLE) and solid-phase extraction (SPE). The primary goal of these extraction techniques is to extract the analytes quantitatively from the analytical matrix. When these techniques are used the analytes are normally collected in 0.2 - 10 ml of extraction solvent.

Quantitative extraction in LLE can only be achieved by using large volumes of extraction solvent relative to the sample volume. For the extraction of a 1 ml sample of a biological fluid 0.5 - 10 ml of extraction solvent is used. In order to obtain enrichment the extract is evaporated and the analytes reconstituted in a smaller amount of solvent.

In SPE the final extraction volume is governed by the bed volume. The bed volume is the amount of solvent required to fill all the internal pores and interstitial spaces of the particles. For a 40 micron, 60 Angstrom sorbents, bed volumes are in the order of 120 μl per 100 mg of sorbent. A 1 ml sample of a biological fluid is normally extracted on 100 mg sorbent. The minimum elution volume required is 2 bed volumes or 0.24 ml of solvent. The consequence is that a maximum of 4 times enrichment is obtainable in SPE of a 1 ml sample. In order to achieve higher enrichments the elution solvent must be evaporated and the analyte reconstituted in a smaller volume of solvent.

Due to practical limitations it is difficult to reconstitute a sample in solvent volumes smaller than 100 μl. When the analyte is reconstituted in 100 μl of solvent, analyte concentration will not exceed 10 times the analyte concentration in a 1 ml sample. Higher enrichments are often necessary to detect trace amounts of analytes by the capillary separation methods. This fact greatly reduces the applicability of the capillary separation methods in bioanalysis.

Micro extraction

A solution to this problem is to apply a micro extraction (ME) technique, in which the analytes are extracted from a large volume of sample solution into a small volume of an acceptor phase. The acceptor phase can either be a solid such as in solid-phase micro extraction (SPME), or an organic solvent such as in liquid-liquid micro extraction (LLME). A large sample volume is used in order to collect quantifiable amounts of the

analytes and enrichment is facilitated by trapping the analytes in the small volume of the acceptor phase. The extraction is carried out until equilibrium.

Figure 1 illustrates the difference between traditional LLE and LLME. A 1 ml sample containing 1 μg/ml of the analyte is extracted into 1 ml of solvent in LLE and into 10 μl of solvent in LLME. The analyte partition coefficient between the aqueous matrix and the organic solvent is 100. Figure 1 shows that 0.99 μg of the analyte is extracted into the organic extract by LLE and that 0.0099 μg remains in the sample. Analyte concentration in the extract is 0.99 μg/ml. In LLME 0.5 μg of the analyte is extracted into the organic acceptor phase while 0.5 μg remains in the sample. Sample concentration in the acceptor phase is 50 μg/ml. This fact demonstrates that very high enrichments are obtainable in LLME without solvent evaporation and reconstitution.

SPME is a well established solvent free sample preparation technique. In SPME the acceptor phase is a solid polymer coated on a fibre. The polymeric acceptor phase is non-volatile and acts as a sorbent for partitioning of the analytes. The volume of the polymeric acceptor phase is less than 1 μl. SPME was originally developed for the analysis of organic compounds in water samples and the method is particularly useful for trace analysis of volatile organic compounds prior to GC analysis. When SPME is applied to bioanalytical samples such as plasma or urine several difficulties are observed. In drug analysis enrichment from a biological matrix is greatly reduced as compared to enrichment from a pure water sample. This is due to reduced capacity of the acceptor phase. In addition the polymeric acceptor phase is easily contaminated when SPME is applied to bioanalysis and cross contamination between samples may easily occur. These facts greatly limit the applicability of SPME in bioanalysis. A solution to this problem is to apply a LLME technique.

Basic principles of LLME

The basic theory of SPME is well documented and is applicable to LLME. If we define the acceptor/sample partition coefficient as K_{as}, the volume of acceptor solution as V_a, the volume of sample solution as V_s and the initial sample concentration as C_0, the amount of analyte trapped in the acceptor phase, n, is shown in equation 1:

1. $$n = \frac{K_{as} V_a V_s C_0}{K_{as} V_a + V_s}$$

The concentration of analyte trapped in the acceptor phase, C_a, is:

2. $$C_a = n/V_a$$

When $K_{as} V_a << V_s$ the amount of analyte collected in the acceptor phase is:

3. $$n = K_{as} V_a C_o \text{ and } C_a = K_{as} C_o.$$

Table 1 shows the equilibrium concentration of the analyte trapped in 0.001 ml - 1 ml of acceptor phase after extraction of a 1 ml sample solution containing 1 µg/ml of the analyte. The partition coefficients are 10,100 ,1000 and ∞.

Table 1. Concentration of analyte trapped in variable volumes of acceptor solution with partition coefficients ranging from 10 - ∞.

Volume of acceptor phase (ml)	Concentration of analyte in the acceptor phase (µg/ml) K = 10	K = 100	K= 1000	∞
0.001	9.9	91	500	1000
0.01	9	50	91	100
0.025	8	28.6	38.5	40
0.05	6.7	16.7	19.6	20
0.1	5	9.1	9.9	10
0.5	1.7	1.9	2.0	2.0
1	0.91	0.99	1	1

The concentrations shown in Table 1 demonstrate that as long as the partition coefficients are high, the enrichments obtained by LLME into 0.001 – 0.05ml of acceptor phase are superior to the enrichments obtained by traditional extraction methods.

There are, however, practical problems related to extraction of biological fluids with small volumes of organic solvents. In biological fluids an emulsion is readily formed during liquid-liquid extraction and small volumes of solvent are easily emulsified. Even after centrifugation small solvent volumes are difficult to collect. Extraction of biological fluids with solvent volumes less than 50 μl is therefore not performed in traditional analytical procedures.

One possible way to solve these problems is to use so-called disposable sponges or to use the devices described in the attached Figures 3 and 4.

Disposable extraction sponges

The problems mentioned above can be solved by disposable extraction sponges. Disposable extraction sponges are used to immobilise 10 - 50 μl of extraction solvent. LLME with extraction sponges is particularly suited for sample preparation of biological fluids prior to GC analysis. Solvent immobilised into extraction sponges eliminates the handling problems encountered with small solvent volumes since immobilised solvents are not emulsified and are easily collected after extraction.

Materials used in the manufacture of extraction sponges should be solvent resistant, porous and compressible. In addition the materials should be sufficiently hydrophobic to immobilise water immiscible solvents. The pore size may range from a few micrometers up to millimeters. Expanded polymers and polymeric foams are particularly suited. Examples of solvent resistant polymers are Teflon, Tefzel, Halar, polyethylene and polypropylene. The size of the polymeric material is cut to fit immobilisation of a predetermined volume of solvent.

LLME with disposable extraction sponges

The extraction sponges are filled into a container with the solvent to be immobilised. The sponges are compressed to remove air trapped in the pores and are thereafter soaked in the solvent. The sponges are then ready for extraction.

The sample solutions are filled into extraction vials. Typical volumes of sample solution are 0.5 - 5 ml. Quantitative analysis is always performed by adding an internal standard to the sample solution. The internal standard is added to the sample prior to the extraction and follows the analyte through all the analytical steps. The internal standard compensates for all fluctuations in the procedure. The chemical nature of the sample is

altered prior to extraction to facilitate analyte extraction into the organic solvent. This involves optimisation of pH and addition of salt.

One solvent sponge with immobilised solvent is removed from the container and added to the extraction vial. Extraction is performed by stirring. Any kind of stirring, for example, a magnetic stir bar, can be used. The extraction is continued until equilibrium (10 - 30 min). The solvent sponge is then removed from the sample vial. Immobilised solvent with the enriched analyte is liberated by compression. Compression can be facilitated in any device suitable for squeezing. For example the sponge can be compressed in a disposable medical syringe equipped with a needle and the liberated solvent is filled into micro sampling vials made to fit into a GC autoinjector.

Sponges able to immobilise 25 µl of solvent are suitable in many applications. As shown in Table 1 enrichments of 30 are obtained for analytes having a partition coefficient of 100. A solvent volume of 25 µl is sufficiently large to allow easy handling. This solvent volume is also large enough to avoid overloading and reduced enrichment during extraction. One sponge is used for each sample and used sponges can be stored safely in a container prior to destruction. Compared to traditional methods for sample preparation, LLME with solvent sponges greatly reduces solvent consumption and hazards to workers and the environment.

The present invention aims to solve the problem introduced above by utilising so-called micro back extraction, referred to above and hereinafter as liquid-liquid-liquid micro extraction (LLLME), to obtain a sufficiently high concentration of the material to be analysed in the acceptor solution.

The principles of LLLME will be explained in more detail below.

Liquid-liquid-liquid micro extraction (LLLME)

Separation techniques used in capillary electrophoresis such as capillary zone electrophoresis, micellar electrokinetic chromatography and capillary electrochromatography favour injection of low ionic strength aqueous samples. Due to the injection of nl volumes of samples, high enrichments are required in bioanalysis of drugs present in trace amounts in biological fluids.

Most drugs are ionic. Ionic organic substances can be enriched by LLLME. The principle of LLLME for isolation of ionic organic molecules is illustrated in Figure 2.

Clean-up and concentration of analytes are based on partitioning of the analytes from a large volume of the aqueous sample matrix through a membrane and into a small volume of an aqueous acceptor phase. The membrane acts as a clean-up barrier between two aqueous phases. Both basic and acid compounds can be enriched with LLLME. The pH of the matrix is adjusted so that the analytes are uncharged. This permits them to pass through the membrane into the aqueous acceptor solution on the other side. The pH of the acceptor solution is adjusted to a pH where the analytes are ionised, thus preventing them from re-entering the membrane. Only small uncharged molecules can pass through the membrane and only molecules which are soluble in the membrane and in the acceptor solution can be enriched. Water soluble neutral substances remain in the matrix. Neutral hydrophobic substances partition into the membrane and not into the acceptor phase. Substances with the opposite charge as the analytes remain in the matrix. LLLME is thus a powerful clean-up technique.

The driving force for the extraction is dependant on the product of the analyte partition coefficients between the membrane and the sample solution and between the acceptor phase and the membrane which is equivalent to the analyte partition coefficient between the acceptor phase and the sample matrix. Compounds having a large partition coefficient between the two aqueous phases will be enriched. This partition coefficient will be large for many drugs. LLLME therefore has the potential to act as both a powerful enrichment and clean-up technique for many ionic drugs.

If we define the acceptor/membrane partition coefficient as K_{al}, the membrane/sample partition coefficient as K_{ls}, the acceptor/sample partition as K_{as}, the acceptor volume as V_a, the membrane volume as V_l, the sample volume as V_s, and the initial sample concentration as C_o, the amount of analyte extracted by LLMBE, n, is:

$$4. \qquad n = \frac{K_{al}\, K_{ls}\, V_a\, V_s\, C_o}{K_{al}\, K_{ls}\, V_a + K_{ls}\, V_l + V_s} = \frac{K_{as}\, V_a\, V_s\, C_o}{K_{as}\, V_a + K_{ls}\, V_l + V_s}$$

In LLLME the membrane volume should be as small as possible. Then $K_{ls}\, V_l$ will be negligible and equation 4 can be reduced to :

$$5. \qquad n = \frac{K_{as}\, V_a\, V_s\, C_o}{K_{as}\, V_a + V_s}$$

Equation 5 can be used to estimate analyte enrichment from a 1 ml sample solution containing 1 μg/ml of the analyte as a function of acceptor phase volume and the partition coefficient. The results obtained are equivalent to the results shown in Table 1 showing that enrichments from a 1 ml sample into 0.001-0.05 ml of acceptor solution are superior to the enrichments obtained by traditional extraction methods.

Many ionic drugs have partition coefficients larger than 100 between two aqueous phases: one having a pH where the drugs are charged and the other a pH where the drugs are uncharged. When an analyte with a partition coefficient of 100 is trapped in 10 μl acceptor solution from 1 ml sample with a concentration of 1 μg/ml, analyte concentration in the acceptor phase is 50 μg/ml. This fact demonstrates that LLLME is able to provide enrichments not obtainable by any other extraction method. LLLME is therefore particularly useful as an extraction technique for modern capillary separation methods such as CE.

The chemical nature of the membrane is important in obtaining short analysis times. Extractions should be continued until equilibrium between the three phases is established. If the membrane/sample partition coefficient is low, equilibrium times will be long and will approach infinity for analytes which are very poorly soluble in the membrane. The solvent forming the membrane should therefore be a good solvent for the target analyte. The chemical nature of the membrane is also important for tuning of the selectivity.

Summary of Inventions

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The present invention aims to improve the known art and to utilise the above suggested possibilities and therefore relates, in a first aspect, to an apparatus for carrying out liquid-liquid micro extraction or liquid-liquid-liquid micro extraction with high enrichment, and the apparatus is characterised in that it comprises

a) a container for a sample solution having volume Vs with dissolved substance, analyte, to be analysed,

b) a second container arranged in the first container, preferably a disposable container, having permeable membrane walls, for an acceptor solution, having volume Va, wherein

   1) $Vs:Va \geq 50$ and

   2) about $1\ \mu l \leq Va \leq 50\ \mu l$,

c) stirring means, preferably a magnetic bar.

In one embodiment the container for the acceptor solution is a microporous hollow fibre, optionally of an active polymer.

As mentioned in the introduction, the invention also relates to methods for extraction and thereby relates, in a first extraction aspect, to a method for liquid-liquid micro extraction with high enrichment by using the above described apparatus, and this method is characterised in that

a) the container for acceptor solution is lowered into an acceptor solution so that the membrane wall is impregnated with, and the container is filled with, a defined volume of the acceptor solution,
b) the container filled under a) is transferred to the container having a defined volume of the sample solution with the analyte that is sought,
c) the sample solution with analyte is stirred until extraction equilibrium is established for the analyte in the two solutions, and
d) the acceptor solution containing enriched analyte is removed from its container for analysis of the analyte.

In a second extraction aspect, the invention relates to a method for liquid-liquid-liquid micro extraction with high enrichment by the use of the apparatus according to claim l, and this method is characterised in that

a) the walls of the container for the acceptor solution are impregnated with, for immobilisation of, a liquid that is immiscible with the sample solution and the acceptor solution,
b) the container for acceptor solution is filled with a defined volume thereof and
c) is lowered into the container having a defined volume of the sample solution with the analyte that is sought,
d) the sample solution with analyte is stirred until extraction equilibrium is established between
    i) the sample solution and the immobilised liquid, and
    ii) the immobilised liquid and the acceptor solution, and
e) the acceptor solution with enriched analyte is removed from its container for analysis of the analyte.

The latter method is particularly suited for enrichment of acidic or basic analytes. For example, basic analytes can be enriched from basic, aqueous, biological samples by

utilising an acceptor liquid in the form of an acidified, aqueous liquid and an organic liquid immobilised in the membrane that is immiscible with the aqueous liquids.

Here, as mentioned previously, it may be advantageous to use a microporous hollow fibre, but it is also possible to use an active polymer.

In a final aspect, as mentioned above, the invention relates to a disposable device for use in liquid-liquid micro extraction, which is characterised in that it has the form of a sponge-like body having a defined pore volume for absorption of an immobilised acceptor solution for an analyte from a volume of a sample solution.

Brief Description of the Drawings

The invention will now be illustrated in more detail with reference to the accompanying drawings where:

- Figure 1 shows a comparison between liquid-liquid extraction and liquid-liquid micro extraction,
- Figure 2 shows the principle for liquid-liquid-liquid micro extraction,
- Figure 3 shows a possible device for utilisation in LLLME, or LLME,
- Figure 4 shows another possible device for LLLME, or LLME,
- Figure 5 shows chromatograms obtained in connection with Example 1, and
- Figure 6 shows electropherograms obtained in connection with Example 2.

Detailed Description of the Invention

Disposable devices for LLLME

Devices for LLLME should accomplish extraction from a large sample volume through a negligible volume of a membrane into a small volume of an aqueous acceptor solution. The membrane should be a thin film with a large surface area. The membrane can either be a solid (liquid-solid-liquid micro extraction, LSLME) or a liquid (liquid-liquid-liquid micro extraction, LLLME).

In LLLME the solvent forming the liquid membrane should be immobilised. Any material able to immobilise a water immiscible solvent can be used. Hydrophobic hollow fibres are particularly useful. The fibres can be made of a polymeric materials such as Teflon, polypropylene or polyethylene. The inner diameter of the hollow fibre is in the range of 0.05 – 1 mm, the wall thickness is typically in the range of 0.01 – 0.3 mm and the average pore size is in the range of 0.01 – 10 μm. The length of the fibre is typically 2 – 10 cm to allow fixed volumes of acceptor solution in the range of 5 – 50 μl to be filled into the hollow fibre.

In LSLME the membrane may be a polymeric film made of a materiale able to sorb uncharged compounds. Examples of such materials are polydimethylsiloxane (PDMS), polyacrylate or polystyrene divinyl benzene. These materials are also used as well known sorbents in SPME. The membrane thickness is preferably 0.5 - 50 μm. The membrane may be supported by a rigid framework and formed into tubes with the same dimensions as the hollow fibres described above.

Devices for LLLME should be disposable. Impurities from one sample may be trapped in the membrane and these impurities may contaminate other samples. One sample pre-treatment device should therefore be used for each sample. Disposable devices should also be connected to commercially available sample preparation vials.

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Disposable devices for LLLME are shown in Figures 3a and 4a. Figures 3b and 4b show the devices connected to sample vials filled with sample solution. The guiding rod can be a stainless steel rod or a rod made of a polymeric material. The top of the guiding rod can be connected to a needle guide to allow filling of the hollow fibre with acceptor solution from a syringe. The hollow fibre in Fig. 3b is connected at both ends to guiding rods.

Sample preparation with disposable LLLME devices

In LSLME the membrane can be used as it is. In LLLME the liquid membrane is formed by dipping the hollow fibre into the organic solvent for 5-30 sec. to allow the solvent to penetrate into the pores of the fibre. Acceptor solution is then filled into the fibre by a syringe. Normally, fibres with an inner tube volume of 10 μl are preferred, since 10 μl of acceptor solution gives high analyte enrichments and 10 μl volumes can be handled with commercially available syringes. The acceptor solution has a pH where the target analytes are charged. Extraction is performed by connecting the LLLME device to the sample vial. The sample filled into the sample vial is buffered to a pH where the analytes are neutral. Typical sample volumes are 0.5 – 5 ml of a biological fluid. An internal standard is always added to the sample solution before extraction to compensate for fluctuations in the procedure. Extraction is performed by stirring, for example with a magnetic stir bar placed in the sample vial. Extraction is continued until equilibrium between the three phases is established. When equilibrium is reached (15-60 min) the acceptor solution is collected with a syringe and filled into autosampler vials for automated injection into the analytical instrument.

The invention will now be illustrated in more detail with the aid of the following examples.

The device shown in Figure 4a was used to demonstate the potential of liquid-liquid micro extraction and liquid-liquid-liquid micro extraction. The hollow fibre used was a polypropylene fibre with pore size 0.2 μm (Accurel PP Q3/2) and was purchased from Akzo Nobel (Wuppertal, Germany). The inner diameter was 600 μm, the wall thickness was 200 μm and the length was 5.5 cm.

Example 1: Liquid - liquid micro extraction (LLME)

LLME is demonstrated by the extraction of 5 nmol/ml sample solutions of diazepam (D) and prazepam (P) prepared in 1.0 M acetate buffer pH 5.5, in urine and in human plasma. A standard solution in octanol (5 nmol/ml) was prepared as a reference solution for direct injection into the gas chromatograph. The pH of the standard solution in urine was adjusted to pH 5.5 before extraction. To an aliquote of plasma (1080 μl) was added 120 μl methanol to reduce the protein binding of the benzodiazepines prior to extraction and the mixture was agitated for 1 min. LLME was accomplished by placing 1.2 ml of the sample solutions in 2 ml autosampler vials (Chromacol, Trumball, CT., USA). The hollow fibre was filled with 10 μl of 1-octanol. After 1 min, to ensure that the solvent would completely penetrate the pores, the hollow fibre was immersed into the autosampler vials. The sample solution was stirred with a magnetic stir bar during extraction. After 30 min 1 μl of octanol was withdrawn from the hollow fibre with a GC syringe and injected into the gas chromatograph. The gas chromatographic separation was achieved on a poly-(dimethylsiloxane) column (30 x 0.25 mm i.D., 0.25 mm film thickness) and the compounds were detected with a nitrogen-phosphorous detector (NPD). Helium (1 ml/min) was used a carrier gas. The chromatographic separation was achieved by temperature programming. The temperature was held at 180 ^{0}C for 1 min and increased at 20 ^{0}C /min to 300^{0}C. Figure 5 shows chromatograms of the reference solution in octanol (5 nmol/ml) and chromatograms of the sample solutions (5 nmol/ml) of diazepam and prazepam in acetate buffer, in urine and plasma after enrichment by LLME. The chromatograms demonstrate preconcentration by a factor of 100 and 70, respectively, for diazepam and prazepam from the acetate buffer, urine and the plasma sample.

Example 2: Liquid-liquid-liquid micro extraction (LLLME)

LLLME is performed with 1-octanol as the immobilised liquid. The hollow fibre was immersed for 5 sec in 1-octanol which is sufficient for 1-octanol to penetrate and fill the

pores of the fibre. 10 µl of 0.1M HCl was used as acceptor solution and was filled into the impregated fibre with a syringe. A standard solution of 4 µg/ml of diphenhydramine in 0.1 M HCl was prepared as a reference for direct injection into the CE instrument. In addition, sample solutions of diphenydramine (4 µg/ml) were prepared in 0.1 M NaOH, in urine and plasma. Before extraction the pH in the urine and plasma sample solutions were adjusted to a pH 12-13 with NaOH. 1.5 ml of the sample solutions were placed in 2 ml autosampler vials. LLLME was accomplished by stirring with a magnetic stir bar for 30 min. The acceptor solution was removed after extraction and analysed by CE. Separations were perfomed inside a 10 cm effective length (52 cm total length) x 50 µm internal diameter fused silica capillary. A 20 mM sodium acetate buffer adjusted to pH 4.5 with acetic acid was utilised as separation buffer. Sample introduction was accomplished by hydrodynamic injection with a pressure of 0.5 psi for 5 sec. Separations were performed at 25 kV, while detection was accomplished at 215 nm. Electropherograms are shown in Figure 6. The electropherograms show that diphenhydramine (DH) was preconcentrated by a factor of 90 from the sample solution prepared in 0.1 M NaOH and in urine. A preconcentration of 50 was achieved from plasma. The lower enrichment from plasma is due to protein binding of the analyte. For both of the biological samples, excellent sample clean-up was observed in addition to analyte enrichment. In spite of the high sample complexity, almost no matrix components were observed in the electropherograms obtained by capillary zone electrophoresis.